

Dysregulation of granulosa bone morphogenetic protein receptor 1B density is associated with reduced ovarian reserve and the age-related decline in human fertility

Article

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1 **Dysregulation of granulosal bone morphogenetic protein**
2 **receptor 1B density is associated with reduced ovarian**
3 **reserve and the age-related decline in human fertility**

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23

24 **Abstract**

25 Reproductive ageing is linked to the depletion of ovarian primordial follicles, which causes
26 an irreversible change to ovarian cellular function and the capacity to reproduce. The current
27 study aimed to profile the expression of bone morphogenetic protein receptor, (BMPRII) in
28 53 IVF patients exhibiting different degrees of primordial follicle depletion. The granulosa
29 cell receptor density was measured in 403 follicles via flow cytometry. A decline in
30 BMPRII density occurred at the time of dominant follicle selection and during the terminal
31 stage of folliculogenesis in the 23-30 y good ovarian reserve patients. The 40+ y poor
32 ovarian reserve patients experienced a reversal of this pattern. The results demonstrate an
33 association between age-induced depletion of the ovarian reserve and BMPRII receptor
34 density at the two critical time points of dominant follicle selection and pre-ovulatory follicle
35 maturation. Dysregulation of BMP receptor signalling may inhibit the normal steroidogenic
36 differentiation required for maturation in older patients.

37

38 **1. Introduction**

39 Reproductive ageing is linked to the declining capacity to regenerate cells and tissues,
40 causing irreversible changes to ovarian cellular dynamics and ultimately reducing the
41 capacity to reproduce. As the average age of fertility-challenged patients climbs towards 40
42 years (y), there is an urgency to characterise the cellular changes that occur in the ovary with
43 time. The response of the ovaries to cyclic recruitment of primordial follicles forms the basis
44 of the clinical documentation of the antral follicle count (AFC) (Almog, et al. 2011). The
45 AFC and age are highly correlated to histologically determined ovarian primordial reserve
46 (Hansen, et al. 2011, van Rooij, et al. 2005). As the primordial follicle reserve declines, the
47 endocrine, paracrine, and autocrine regulation adapts to a changing environment. It is this
48 changing landscape that requires further investigation to provide an alternative treatment to
49 preserve the primordial follicles, and to adjust the cellular regulation to achieve oocyte
50 competence and improve fertility rates in older patients.

51

52 Earlier research has highlighted the potential role of bone morphogenetic protein (BMP)
53 signalling in regulating ovulation rate in sheep (Campbell, et al. 2006, Galloway, et al. 2000,
54 Juengel, et al. 2011), and has led us to further investigate the molecular regulation of
55 folliculogenesis by the BMPs (Regan, et al. 2015, Ruoss, et al. 2009). During a natural cycle,
56 small antral follicles with sufficient granulosa follicle-stimulating hormone receptor (FSHR)
57 expression are recruited in response to the intercycle rise in FSH, and one of these is
58 subsequently selected to become the dominant follicle. Follicles with reduced FSHR and
59 luteinising hormone receptor (LHR) become less responsive as the dependence from
60 pituitary FSH stimulation shifts to LH, and circulating FSH concentrations decline (Lapolt,
61 et al. 1990, Xu, et al. 1995, Zeleznik, et al. 1974). These subordinate follicles are destined
62 for atresia. The selected dominant follicle is the one with greatest gonadotrophin

63 responsiveness, and is dependent on the acquisition of FSHR-induced LHR by granulosa
64 cells; and this follicle continues growing to the ovulatory stage.

65

66 Previous research has shown that, at the time of declining FSH levels, a reduction in BMP6
67 (Erickson and Shimasaki 2003), BMP15 (Feary, et al. 2007) and the type 1 TGF β
68 superfamily receptor BMPR1B (Feary, et al. 2007, Regan, et al. 2015), occurs after
69 dominant follicle selection. Once selected, follicle growth and cellular proliferation
70 continues, leading to an increased oestrogen and inhibin production by the granulosa cells.
71 Together, these hormones suppress pituitary FSH output further, ensuring the demise of
72 subordinate follicles. When the threshold for oestrogen's positive feedback action on the
73 hypothalamus-pituitary axis is met, preparation for the ovulation phase begins with a
74 cessation of cell proliferation, and early luteinisation changes taking place. During cellular
75 and steroidogenic differentiation, activin (Young, et al. 2012), insulin-like peptide 3 (INSL3)
76 (Anand-Ivell, et al. 2013), anti-mullerian hormone (AMH) (Andersen, et al. 2010, Ogura
77 Nose, et al. 2012, Weenen, et al. 2004) and gonadotrophin surge attenuating factor (GnSAF)
78 activity (Martinez, et al. 2002) declines, from dominant follicle selection to the termination
79 of folliculogenesis at ovulation.

80

81 The functional role of BMPR1B receptor in follicle development has received considerable
82 attention in recent years following the discovery that hyper-prolific sheep with the Booroola
83 (FecB) phenotype have a naturally occurring mutation in the kinase domain of BMPR1B that
84 perturbs antral follicle development and ovulation rate (Souza et al 2001; Mulsant et al
85 2001). The BMP ligands, 2, 4, 6, 7, and 15 form a receptor-ligand complex with the type 1
86 TGF β receptor BMPR1B, and recruit the type 2 TGF β receptor BMPR2. The complex
87 initiates phosphorylation of the intracellular substrate molecules, which are the receptor-
88 regulated Smads. The Smad forms a complex with a common mediator, Smad 4, and

89 translocates to the nucleus where transcription of BMP-responsive genes takes place. Smad
90 signalling is modulated by repressor and activator molecules in the nucleus, cytoplasm, and
91 in the extracellular matrix. Alternatively, BMPs activate the non-Smad pathway mitogen-
92 activated protein kinase (MAPK) such as extracellular signal-regulated kinase (ERK 1/2) or
93 Ark (Inagaki, et al. 2009, Ryan, et al. 2008)

94

95 In the ovary, granulosa cell signalling induced by the gonadotrophins, FSH and LH, activate
96 the FSHR or LHR and stimulate cAMP-PKA, which increases the CYP19A1 aromatase to
97 facilitate oestrogen synthesis. Progesterone synthesis is inhibited by the suppression of
98 steroidogenic regulatory protein (StAR) (Abdo, et al. 2008, Pierre, et al. 2004, Tajima, et al.
99 2003, Val, et al. 2003), which is essential for progesterone synthesis in the granulosa cell
100 (Moore, et al. 2001). Alternatively, or in addition, BMPs inhibit ERK 1/2 signalling, which
101 provides inhibitory control over the balance of progesterone and oestrogen (Miyoshi, et al.
102 2007, Nakamura, et al. 2012, Ogura Nose, et al. 2012).

103

104 Given the particular focus of interest on BMPR1B in ovarian function, the current study
105 aimed to comprehensively profile the expression of granulosa BMPR1B in a range of
106 patients, of different ages and stages of ovarian primordial follicle depletion, who were
107 receiving treatment for infertility. Previous reports documenting ovarian BMPR1B
108 expression have evaluated expression at the mRNA level in pooled follicles from different
109 size classes (Chen, et al. 2009, Estienne, et al. 2015). However, mRNA expression does not
110 necessarily reflect expression of translated functional BMPR1B protein on the cell surface.
111 In contrast, in this study we collected an average of ~ 8000 granulosa cells from each
112 individual follicle over a comprehensive range of follicle diameters from 4 mm to 27 mm.
113 Immunofluorescent labelling and flow cytometry were then used to measure the granulosa
114 cell surface-expressed mature receptor protein density for the BMPR1B receptor.

2. Materials and Methods

2.1. Patients

A total of 401 follicles were collected from 53 patients undergoing standard fertility treatment previously reported in accordance with the PIVET Medical Centre Algorithm, and are presented in Table 1 (Yovich, et al. 2012). Follicles were collected irrespective of previous aetiology, but limited to exclude unusual medical conditions, hormonal dysfunction, and polycystic ovarian syndrome; patients were aged between 23 and 45 y. One patient, out of three patients undergoing risk reduction removal of the uterus and ovaries, was selected to represent an unstimulated natural healthy cycle prior to the LH surge, and was recruited from King Edward Memorial Hospital (KEMH).

Table 1 Patient ovarian reserve, based on antral follicle count (AFC) and the number of follicles collected per group.

Ovarian reserve measured indirectly by the Antral Follicle Count (AFC). AFC is the number of follicles between 2-10 mm on day 2-5 of a cycle: A+ = 30-39 follicles; A = 20-29; B = 13-19; C = 9-12, D = 5-8; E = ≤4.

AGE	IVF	Total		Major	Number of Follicles Collected Per Group							
Year	Patient	Follicle	AFC	Group	Sub		Sub		Sub		Sub	
					Group	#	Group	#	Group	#	Group	#
21-30	8	95	20-40	A+ & A	A+	31	A	64	C	6		
31-34	11	86	13-29	A & B	A	60	B	26	C	17		
35-39	16	102	9-19	B & C	B	50	C	16	D	30	E	6
40-45	18	118	3-8	D & E	D	59	E	19	B	34		
40	1	Natural Cycle Healthy			D	2						

OVARIAN RESERVE GOOD A+ = 30-39 A = 20-29 B = 13-19 C = 9-12 D = 5-8 E = ≤4 POOR

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131

2.2. Human IVF: Ovarian stimulation, follicular fluid and oocyte

Patient treatment consisted of two types of GnRH-LH suppression in conjunction with rFSH, from cycle day 2 for ~ 10 days (Puregon or Gonal F). A GnRH antagonist treatment (Cetrotide) (0.25 µg/day) was administered from day seven until ovulation induction.

136 Alternatively, a GnRH flare agonist treatment (Lucrin) (0.25 µg/day) was administered in
137 conjunction with rFSH on day 2, Ovulation was triggered with either 10 000 IU hCG derived
138 from a urinary preparation (Pregnyl) or a pituitary derived analogue to LH (Ovidrel). Oocyte
139 retrieval was scheduled for 36 hours post-trigger, by transvaginal oocyte aspiration (Yovich
140 and Stanger 2010).

141 *2.3. Antral follicle count*

142 Patients received rFSH based on the patient's profile of age and AFC, to predict the rFSH
143 dose required to stimulate multiple pre-ovulatory follicles (Yovich, et al. 2012). The dose of
144 rFSH was then adjusted to the patient's ovarian response to stimulation. Considerable
145 overlap in rFSH dose was present between age groups, which allowed for a rFSH dose
146 comparison between different ovarian reserve patient groups of the same age. Ovarian
147 reserve was measured indirectly by the antral follicle count (AFC) (Hansen, et al. 2011).
148 AFC was defined as the number of follicles between 2-10 mm in size that are present on day
149 2-5 of a cycle. Determination of AFC was ascertained by transvaginal ultrasound and
150 patients were divided into groups accordingly: Group A+ = 30-39; group A = 20-29; group B
151 = 13-19; group C = 9-12, group D = 5-8; group E = ≤ 4. The groups were established based
152 on ovarian response to gonadotrophin hormone stimulation during IVF cycles. (Yovich, et
153 al. 2012).

154 *2.4. Collection of granulosa cells*

155 The diameter of the follicle was calculated using ultrasonography before the clinical
156 aspiration of individual follicles. The first aspiration was collected without flush medium
157 into a test tube, and handed to the embryologist to locate the oocyte and attached cumulus
158 cells if present. Further flushing of the follicle (Quinn's Advantage with Hepes, Sage Media,
159 Pasadena, California) at ~ 1.24-1.72 MPa removed the loosely attached layers of granulosa
160 cells. Once the oocyte was located and removed, the clinician proceeded to the next follicle
161 and repeated the process. The follicular fluid and flush was then layered onto a ficoll density

162 gradient (555485; BD Biosciences, Perth, Australia) and centrifuged to isolate the granulosa
163 cells

164 *2.5. Natural healthy unstimulated cycle collection*

165 The natural cycle patients scheduled for risk reduction removal of the reproductive organs
166 was timed to coincide with day 12 of the menstrual cycle (Table 1). Before removal,
167 ultrasound confirmation of the size of the follicles and the number of follicles present was
168 made. After removal of the uterus and ovaries, the whole follicle was excised and
169 transported to the laboratory. The collection of follicular fluid, isolation of the granulosa
170 cells, and the analysis was performed as described above and below.

171 *2.6. Immunolabelling of granulosa cells*

172 Aliquots of suspended granulosa cells (1×10^6 cells in 100 μ l) were immunolabelled using a
173 double-indirect method as previously described (Abir, et al. 2008, Cai, et al. 2007, Gao, et al.
174 2007). The cells were incubated separately with an optimised concentration of 4 μ g/ml
175 affinity purified polyclonal antibody to goat BMPR1B (sc-5679), (Santa Cruz
176 Biotechnology, Santa Cruz, CA, USA), for 25 min at 5°C; washed with PBS and then
177 incubated with a second antibody, donkey anti-goat conjugated to the fluoro-chrome Alexa
178 488 (Al-Sammerria and Almahbobi 2014). The cells were washed again with PBS and
179 centrifuged at 300 g at 5°C for 5 min. In addition, these antibodies have been used previously
180 in human studies (Abir, et al. 2008, Haÿ, et al. 2004), including flow cytometry analyses
181 (Gao, et al. 2007, Regan, et al. 2015, Whiteman, et al. 1991).

182

183 The routinely used monoclonal antibody against CD45 was added to BMPR1B antibody-
184 containing tubes to enable the subtraction of leukocyte common antigen-positive cells (~
185 3%) not removed during isolation of the granulosa cells using the ficoll density gradient.
186 Unstained samples or the substitution of primary antibody with pre-immune goat IgG (Fig.
187 1A) (Millennium Science, Surrey Hills, Victoria Australia) at the same concentration of the

primary antibody served as a negative control for auto-fluorescence; and a blocking peptide for BMPR1B also confirmed binding specificity (Fig 1B), (sc-5679P; Millennium Science, Surrey Hills, Victoria Australia) and as previously published (Abir, et al. 2008, Al-Samerria and Almahbobi 2014, Haÿ, et al. 2004, Regan, et al. 2015, Weall, et al. 2014)

In the current study, the ‘normal’ goat IgG and unstained control cells emitted an average MFI that was very similar for each individual follicle but different between follicles and patients; therefore, to optimise accuracy, the auto-fluorescence and the nonspecific binding determined by the unstained control for each follicle, was subtracted from each individual follicle. The data were analysed using FlowJo software (Tree Star Inc., Oregon, USA).

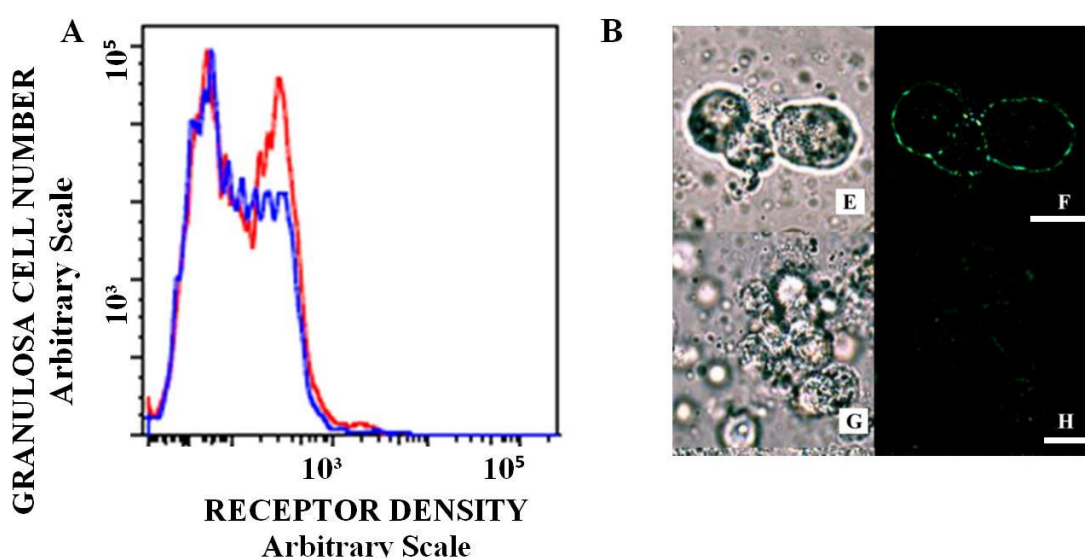


Fig. 1 Validation of immunofluorescent labelling.

A. Unstained control (blue) compared to IgG Isotope control (red) for nonspecific binding and auto-fluorescence. B. Live human granulosa luteal cells with positive fluorescence for BMPR1B (EF), and negative blocking agent for BMPR1B (GH). Bar 10 μ m.

2.7. Fluorescent microscopy

Re-suspended 10 μ l aliquots of BMPR1B immunolabelled, live granulosa cells were placed on slides and visualized using an Olympus DP 70 camera fitted to a Olympus BX-51 upright fluorescent microscope with a 40x UPlan N 0.4 N.A. objective; (Olympus Imaging Australia, Macquarie Park, Australia), (Fig.1B). The granulosa cell slides were allowed to air

dry to reduce movement during digital capture, which would account for the more clumpy appearance compared to the more typical single granulosa cells analysed by flow cytometry. Fluorescent microscopy revealed a positive staining of the cell membrane-bound BMPR1B, as an intermittent, bright, ring-like pattern around the cells. All control samples showed negative staining. Granulosa cells ranged from 8 μm to 25 μm , with the average being 15 μm .

2.8. Flow cytometry

Selective gating of the whole sample to identify a pure granulosa cell population was achieved by graphing forward scatter to remove doublets (FSC-H versus FSC-A) (Regan, et al. 2015). Then Alexa Fluor 488 fluorescent intensity was plotted against Allophycocyanin (APC) intensity to identify and subtract the cells positive for the leukocyte common antigen antibody CD45, which emits in the APC spectrum (Fig. 2A). Auto-fluorescence and nonspecific binding were identified by the unstained sample control BMPR1B expression, and subtracted from the measurement (Fig. 2B).

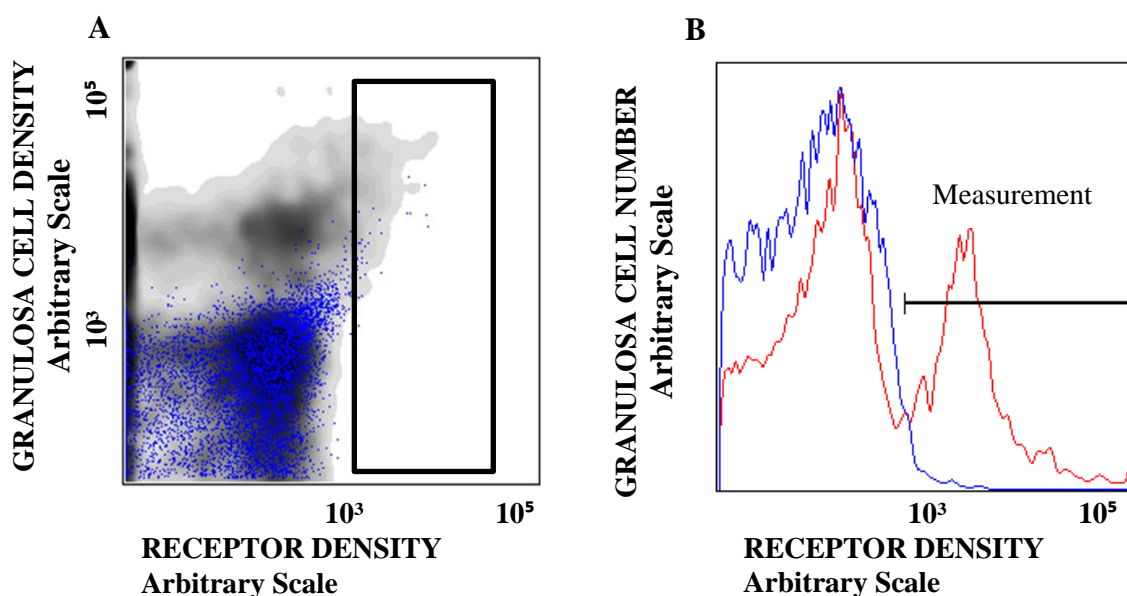


Fig. 2 Validation of gating to measure average receptor density in flow cytometry.

A. Unstained control granulosa cells, represented as blue dots (auto-fluorescence) and immunostained granulosa cells (grey). A rectangle subtraction gate for the leukocyte common antigen CD45 positive cells. B. Subtraction of nonspecific binding and auto-fluorescence at 10^3 ; mean granulosa cell fluorescent intensity (MFI) measurement.

228 2.9. Statistics

229 Mean fluorescent intensity (MFI) was obtained using ~8000 granulosa cells per individual
 230 follicle for the direct measurement of receptor protein expression. The data were subjected to
 231 statistical verification using one-way ANOVA with an uncorrected Fisher's LSD for
 232 follicular size using GraphPad Prism 6. Values in graphs are means \pm S.E.M., and
 233 differences were considered significant if * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, and
 234 **** $p < 0.001$. The letter, such as 'a', signifies a statistical difference to the matching letter,
 235 and an attached asterisk (a*) indicates the significance level for that follicle size category.

236 2.10. Human Ethics

237 Informed consent was obtained from 57 patients undergoing standard fertility treatment at
 238 PIVET fertility clinic Perth, Australia, and three patients undergoing risk reduction removal
 239 of the uterus and ovaries which were recruited from King Edward Memorial Hospital
 240 (KEMH) Perth, Australia. Approval by the Human Research Ethics Committee of Curtin
 241 University of Technology and KEMH Women and Newborn Health Service ethics
 242 committee (WNHS) was obtained for this study.

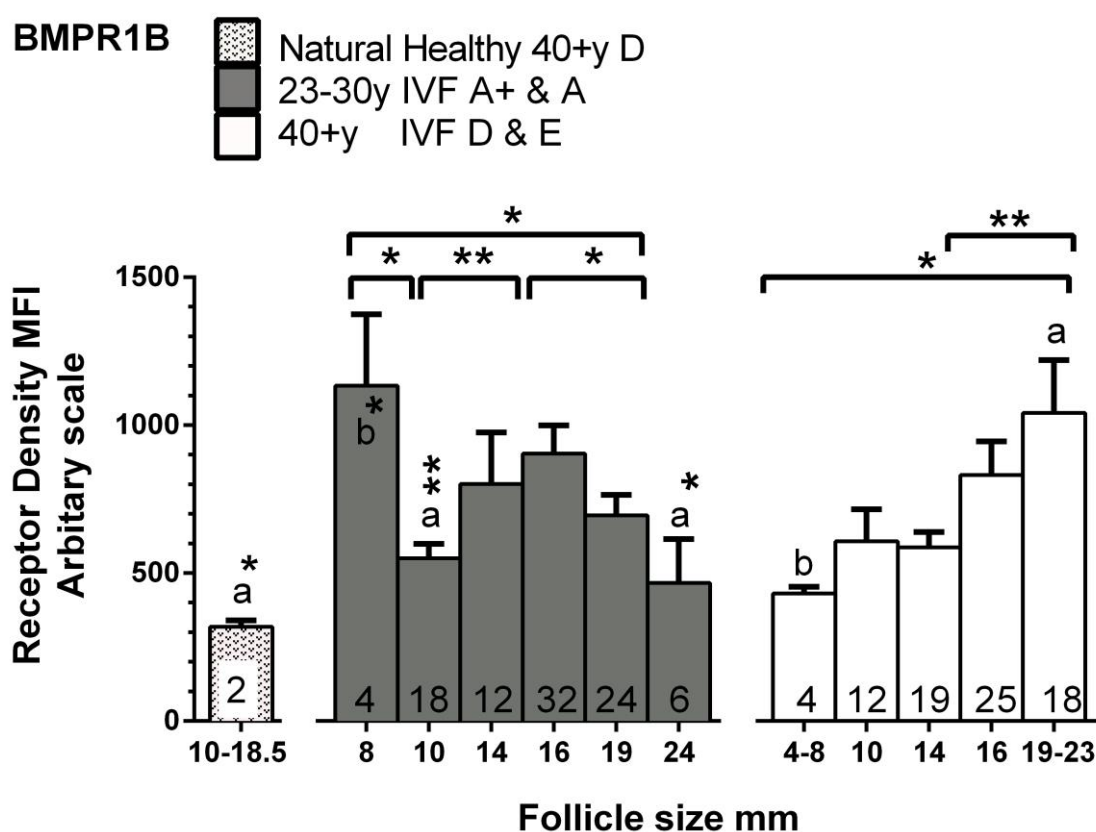
243 3. Results

244 3.1. Follicle development and ovarian reserve

245 Relative to older (40+y) patients, the level of granulosa BMPR1B expression was lower in
 246 the 23-30 y IVF patients in combined AFC groups A+ & A, and showed a biphasic receptor
 247 density pattern (Fig. 3). The biphasic pattern of receptor density consisted of an initial
 248 decrease in BMPR1B in follicles from 8 mm to 10 mm ($p < 0.0201$), followed by an up-
 249 regulation in the follicles to 16 mm ($p < 0.0084$), which was further followed by a significant
 250 decline in follicles to the terminal-end of folliculogenesis of 24-26 mm ($p < 0.0301$, Fig. 3). In
 251 marked contrast, the BMPR1B density increased with follicular size ($p < 0.0044$) in a
 252 monophasic reversed profile in the 40+ y group (Fig. 3). The level of receptor density in the

253 small antral follicles of 8 mm was greater in the young patients than the older patients
 254 ($p < 0.0405$).

255 In a natural healthy cycle, the granulosa cells were collected from a healthy 40+ y with a
 256 group D AFC. When combined, the two follicles of 10 mm and 18.5 mm had a significantly
 257 lower density of BMPR1B compared to the largest follicles of the 40+ y IVF patients with a
 258 group D & E AFC (Fig. 3). The receptor level in the natural cycle was not significantly
 259 different to the level recorded in the younger patients and provides a baseline comparison.



OVARIAN RESERVE GOOD A+ = 30-39 A = 20-29 B = 13-19 C = 9-12 D = 5-8 E = ≤4 POOR

260 with the limitation of reduced interpretation.

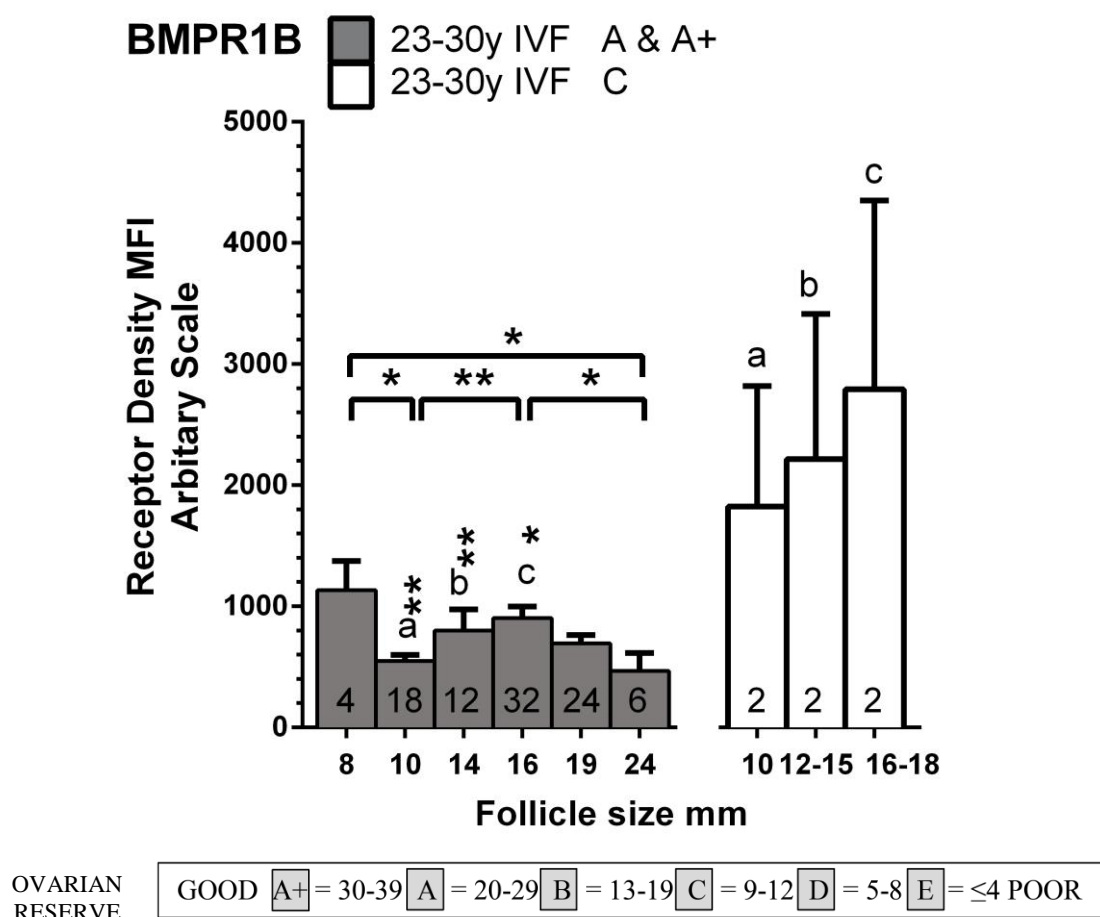
261
 262 **Fig. 3** Granulosa BMPR1B density from follicles of different sizes collected from young
 263 and older IVF patients compared to an unstimulated natural healthy cycle.

264
 265 Granulosa BMPR1B protein density and follicle size profile of a natural healthy
 266 unstimulated patient of 41y with an AFC of D, before the LH surge (patterned bar). Patients,
 267 23-30 y stimulated, IVF cycle with an AFC of A+ & A, (grey bar). Patients, 40+ y

268 stimulated IVF cycle with an AFC of D & E, (white bar). IVF patients were grouped
269 according to ovarian reserve measured indirectly by the antral follicle count (AFC). Mean
270 fluorescent intensity (MFI) was obtained using an average of ~ 8000 granulosa cells per
271 follicle for the direct measurement of receptor protein expression. The data were subjected to
272 statistical verification using one-way ANOVA with an uncorrected Fisher's LSD. Values in
273 graphs are means \pm S.E.M., and differences were considered significant if * $p < 0.05$ and
274 ** $p < 0.01$. The letter, 'a' signifies a statistical difference to the matching letter with an
275 attached asterisk(s) (a*, a**). The number within the column represents the number of
276 follicles analysed for that group.
277

278 *3.2. Dysregulation of BMPR1B receptor density young patients with poor ovarian*
 279 *reserve*

280 In the youngest age group 23-30 y, the majority of the patients had an AFC within groups
 281 A+ & A (Fig. 4). There was no significant difference between the A+ group and the A group.
 282 In contrast, the follicles from the C group patients with low AFC had increased BMPR1B
 283 expression compared to the similar size follicles in the A+ & A group ($p < 0.05$ to $p < 0.001$,
 284 Fig. 4), similar to the profile of the 40+y E AFC patients. Young patients with a very poor
 285 ovarian reserve (group C) for their age do not typically have many follicles available for
 286 collection. The rFSH dose administered ranged from 87 IU to 150 IU in the A+ & A group,
 287 and was 190 IU in the C group.



288 **Fig. 4** Granulosal BMPR1B density and ovarian reserve depletion in 23-30 year-old patients.
 289
 290 Patients were grouped according to ovarian reserve measured indirectly by the antral follicle
 291 count (AFC). AFC is the number of follicles between 2-10 mm on day 2-5 of a cycle. The
 292 data were subjected to statistical verification using one-way ANOVA with an uncorrected

293 Fisher's LSD. Values are means \pm S.E.M., and differences were considered significant if
294 * $p < 0.05$. The letter, such as 'a' signifies a statistical difference to the matching letter, and an
295 attached asterisk (a*) which indicates the significance level. The number within the column
296 represents the number of follicles analysed for that group.

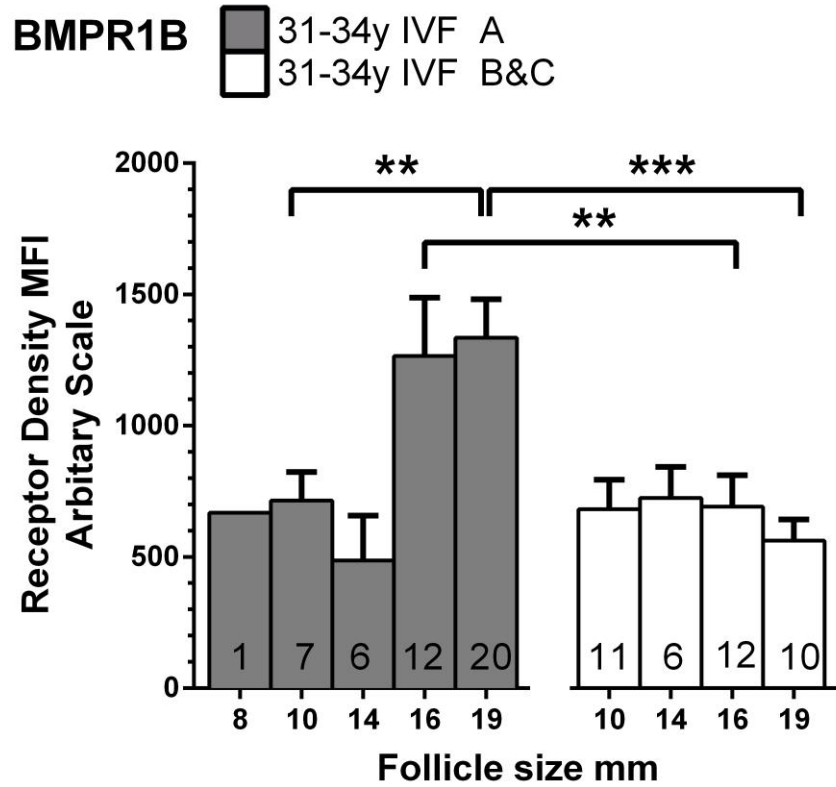
297

298 *3.3. Association between AFC and BMPR1B receptor density in older (31-34y)*
299 *patients*

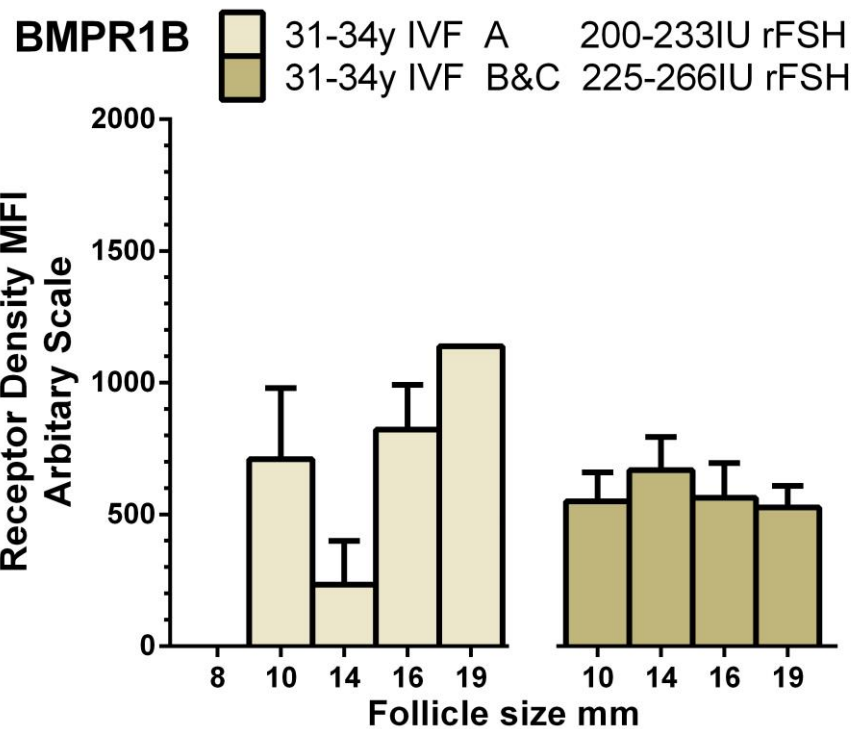
300 In the 31-34 y age group, the decline in ovarian reserve was associated with a loss of
301 receptor density of BMPR1B in the granulosa cells from a peak in the 19 mm follicles in the
302 A group to a significantly lower value in the B and C groups ($p < 0.002$, Fig. 5A). The rFSH
303 dose given ranged from 83.5-266 IU, and when the AFC group comparison was restricted to
304 those patients who received a comparable rFSH dose (200-233 IU), a similar BMPR1B
305 receptor density profile was observed in the B and C group patients. However, in group A,
306 the profile was different in the 16-19 mm follicles, which showed reduced receptor density
307 (Fig. 5B).

308

A



B



OVARIAN
RESERVE

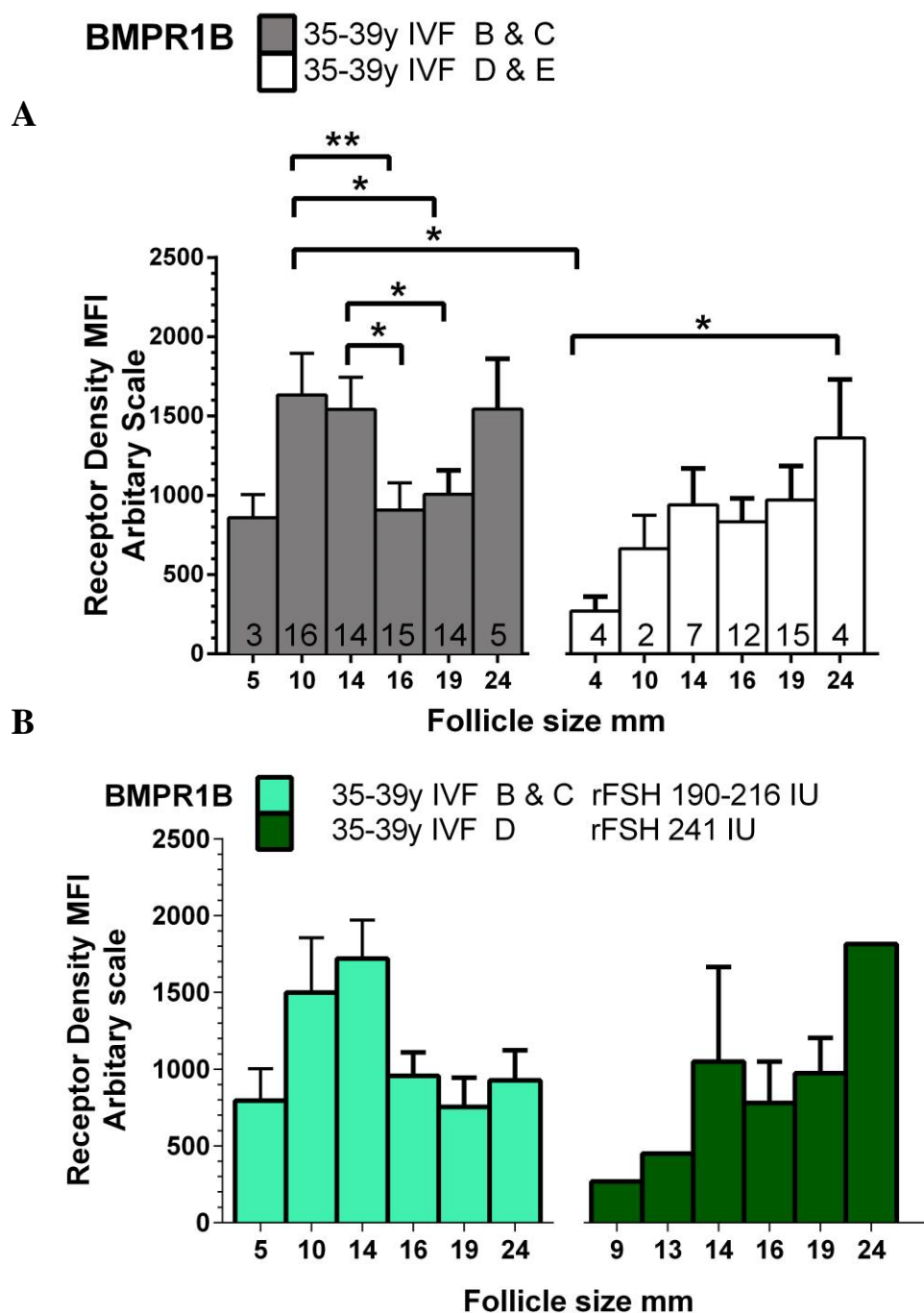
GOOD A+ = 30-39 A = 20-29 B = 13-19 C = 9-12 D = 5-8 E = ≤4 POOR

310
 311 A. Patients were grouped according to ovarian reserve measured indirectly by the antral
 312 follicle count (AFC). AFC is the number of follicles between 2-10 mm on day 2-5 of a cycle.
 313 B. As above, patients were grouped according to AFC but only those who received an
 314 equivalent rFSH dose were included in the analysis. Data were subjected to statistical
 315 verification using one-way ANOVA with an uncorrected Fisher's LSD. Values are means \pm
 316 S.E.M., and differences were considered significant if $*p < 0.05$. The letter, such as 'a'
 317 signifies a statistical difference to the matching letter with an attached asterisk (a*) which
 318 indicates the significance level (ie. 'a' is s.d. to all a*). The number within the column
 319 represents the number of follicles analysed for that group.

320

321 *3.5. BMPR1B receptor density in older patients with declining AFC*

322 The 35-39 y combined B & C group demonstrated a significant reduction in BMPR1B
 323 receptor density in the 10 mm to 16 mm follicles ($p=0.007$), similar to the youngest age
 324 group. With a further decline of the ovarian reserve (group D & E) the receptor density in the
 325 smaller follicles was reduced followed by a steady increase with increasing follicular size
 326 similar to the oldest patients monophasic profile, ($p=0.037$), Fig. 6A). The rFSH dose given
 327 ranged from 83.5-600 IU, and when the AFC group comparison was restricted to those
 328 patients who received a similar FSH dose (190-241 IU), a very similar BMPR1B receptor
 329 density profile was observed (Fig. 6B).



OVARIAN RESERVE

GOOD	A+ = 30-39	A = 20-29	B = 13-19	C = 9-12	D = 5-8	E = ≤4 POOR
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Fig. 6 Granulosal BMPR1B density and ovarian reserve depletion in 35-39 year-old patients.

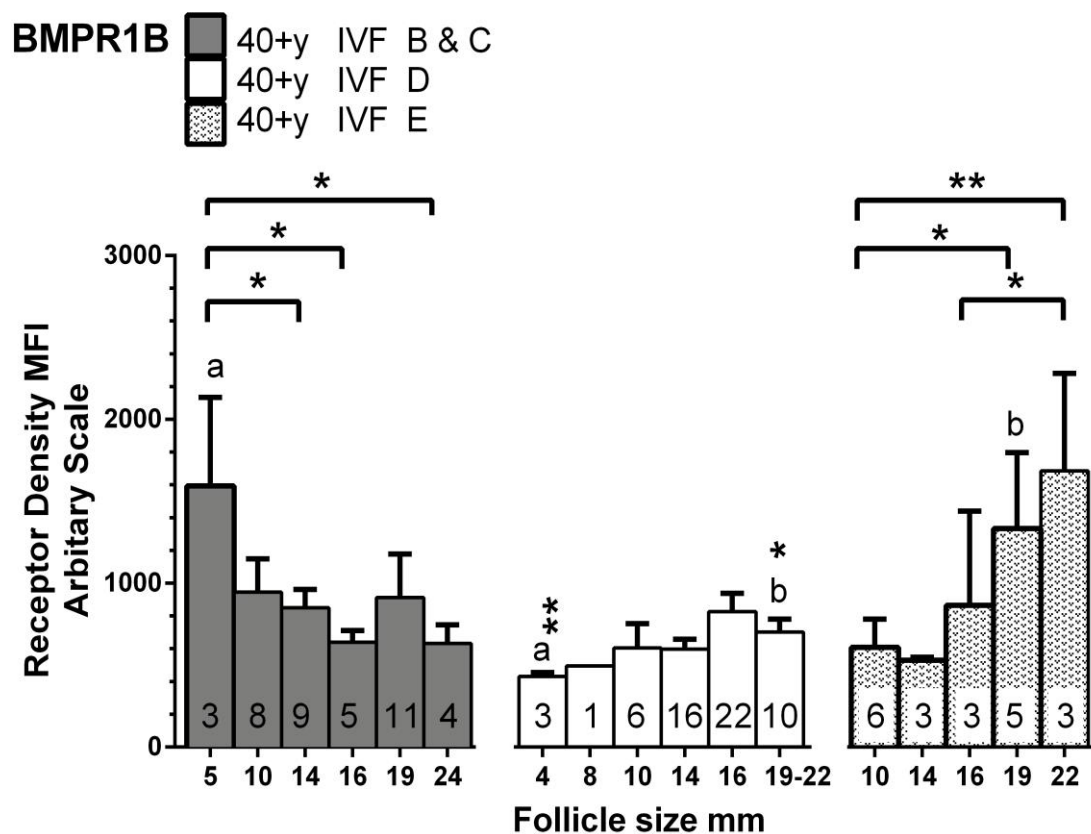
A. Patients were grouped according to ovarian reserve measured indirectly by the antral follicle count (AFC). AFC is the number of follicles between 2-10 mm on day 2-5 of a cycle.

B. As above, patients were grouped according to AFC but only those who received a similar rFSH dose (190-241 IU) were included in the analysis. Data were subjected to statistical verification using one-way ANOVA with an uncorrected Fisher's LSD. Values are means ±

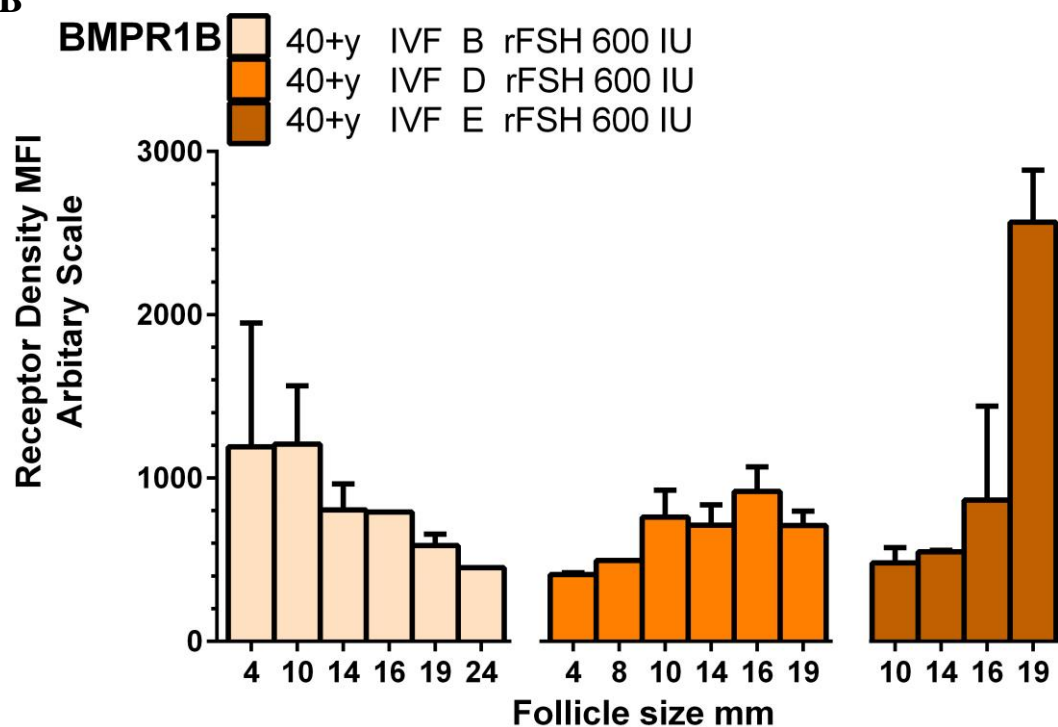
337 S.E.M., and differences were considered significant if $*p < 0.05$. The number within the
338 column represents the number of follicles analysed for that group.

339 The 40+ y patients (40-45 year-old) ranged in AFC from group B to group E (Fig. 7A). The
340 group B & C patients combined demonstrate a higher BMPR1B receptor density in the small
341 follicles, followed by significant down-regulation of receptors as follicle size increased
342 ($p < 0.0176$). With a decline in the ovarian reserve to group D, the receptor density in the
343 smaller follicles was reduced compared to group B ($p < 0.0059$). With a further decline of
344 ovarian reserve from D to group E, the receptor density significantly increased (19 mm
345 follicles, $p < 0.03$); and within the E group increased with follicle size (10 mm to 22 mm,
346 $p < 0.0058$, Fig. 7A). This was similar to the ageing effect observed in the youngest group C
347 patients (Fig 4). The rFSH dose given ranged from 300-600 IU, and when the AFC group
348 comparison was restricted to those patients who received an identical FSH dose (600 IU), a
349 very similar BMPR1B receptor density profile was observed (Fig. 7B).

A



B



OVARIAN
RESERVE

GOOD A+ = 30-39 A = 20-29 B = 13-19 C = 9-12 D = 5-8 E = ≤4 POOR

Fig. 7 Granulosal BMPR1B density and ovarian reserve depletion in 40+ year-old patients.

351

352 A. Patients were grouped according to ovarian reserve measured indirectly by the antral
353 follicle count (AFC). AFC is the number of follicles between 2-10 mm on day 2-5 of a cycle.

354 B. As above, patients were grouped according to AFC but only those who received an

equivalent rFSH dose (600IU) were included in the analysis. Data were subjected to statistical verification using one-way ANOVA with an uncorrected Fisher's LSD. Values are means \pm S.E.M., and differences were considered significant if * $p < 0.05$ and ** $p < 0.01$. The letter, such as 'a' signifies a statistical difference to the matching letter, and an attached asterisk (a*) which indicates the significance level (ie. 'a' is s.d. to all a*). The number within the column represents the number of follicles analysed for that group.

361

362 **4. Discussion**

A continuous process of activation of primordial follicles in the ovary leads to the inevitable depletion of the ovarian reserve in women (Almog, et al. 2011). The gradual decline in ovarian reserve can be indirectly measured by the number of small antral follicles at the beginning of a cycle, which is termed the AFC (Hansen, et al. 2011). The response of the ovary to exogenous gonadotrophins used to treat infertility declines with age, which is strongly correlated to the ovarian reserve (Hansen, et al. 2011). Patients with a poor ovarian reserve are treated with increasing doses of rFSH in an attempt to increase the number of small antral follicles with sufficient FSHR to develop into pre-ovulatory follicles.

371

In sheep carrying the Booroola (FecB) mutation, follicle development was perturbed and ovulation rate increased as a consequence of a point mutation in the BMPR1B gene (Regan, et al. 2015). Therefore, the potential role of BMPR1B within the context of follicle development, ovarian ageing, and fertility in humans is of considerable interest. In the present study, the density of expression of mature cell surface protein for BMPR1B was measured by flow cytometric analysis. We found that a reduction in the number of growing follicles was linked to the sequential disruption of BMPR1B density on the surface of granulosa cells.

380

Ovarian reserve depletes sequentially with age in a slow continuous process. The results in this study show a gradual degradation in the density of receptors, which is perpetuated

383 through the different age groups. The change observed may appear difficult to interpret;
384 however, within each age group, there is evidence of over-expression followed by a
385 weakness in expression, a lack of down-regulation, and eventually, reduced receptor density,
386 ultimately leading to increased levels of BMPR1B in the largest follicles of the oldest,
387 poorest ovarian patient groups.

388

389 An important finding of this study was that a decline in granulosa BMPR1B receptor
390 density occurs in follicles of a size that would correspond to the time of cyclic dominant
391 follicle selection, and again in the largest follicles from the best prognosis IVF patients, aged
392 23-30 y. In comparison, the older, 40+ y, poor ovarian reserve patients exhibited a reversal
393 of this pattern (Fig. 3). The emergence of the dominant follicle in a natural cycle and the
394 multiple 'dominant' follicle cohort of an IVF stimulated cycle occur at the same follicular
395 size (Baerwald, et al. 2003, Baerwald, et al. 2009). The growth rate of ovulatory follicles
396 from a major wave was also not significantly different in an IVF cycle (Baerwald, et al.
397 2009). The rFSH only extends the window of recruitment that promotes multiple dominant
398 follicles (Baird 1987, Fauser and Van Heusden 1997). Therefore, it is possible to compare
399 the receptor density to the physiological process indicated by the size of the follicle, such as
400 dominant follicle selection. It is, therefore, speculated that enhanced BMP signalling arising
401 from elevated pre-ovulatory BMPR1B levels would inhibit the normal steroidogenic
402 differentiation required for maturation of the follicle in older patients with reduced AFC. A
403 decrease in small antral follicle number has been associated with a rise in luteal and start-of-
404 cycle FSH and LH with a corresponding decrease in inhibin B, AMH, and IGF 1 (Klein, et al.
405 2000, Pal, et al. 2010). The increase in FSH and LH has been shown to accelerate the early
406 growth of small follicles, followed by reduced growth rates of the pre-ovulatory follicles in
407 older patients. Other ovarian age related changes were associated with an increase in
408 mitochondrial deletions in granulosa cells (Seifer, et al. 2002) and an increase in the number
409 of chromosomal errors (Handyside, et al. 2012).

410 *4.1. BMPR1B down-regulation and dominant follicle selection*

411 The biphasic down-regulation of the density of the TGF β superfamily type I receptor,
 412 BMPR1B, during folliculogenesis was similar to our previous finding in unstimulated young
 413 adult sheep (Regan, et al. 2015). During dominant follicle selection in sheep, and at an
 414 equivalent size in gonadotrophin stimulated humans, granulosa expression of BMPR1B was
 415 reduced, followed by a sequential increase with follicle size (Fig 3). The similarity between
 416 the sheep in natural cycles and the human IVF model suggests that rFSH has minimal impact
 417 on receptor expression levels and on the timing of dominant follicle selection. The addition
 418 of gonadotrophin in the form of rFSH masks the normal physiological pituitary drop in FSH,
 419 allowing a prolonged recruitment phase that enables multiple follicles to grow (Rice, et al.
 420 2007). The process of recruitment and dominant follicle selection should therefore be
 421 comparable to a normal unstimulated IVF cycle.

422

423 In other studies, granulosa cell expression of BMPR1B has been shown to increase with
 424 follicle size (Chen, et al. 2009, Estienne, et al. 2015), which is consistent with our findings.
 425 However, the pre-ovulatory, leading dominant follicle in sheep was pooled with smaller
 426 follicles in these studies, which would effectively mask the down-regulation (Regan, et al.
 427 2015). The down-regulation of granulosa cell BMPR1B expression in the present study was
 428 consistent with findings for sheep dominant follicles compared to the subordinate follicles
 429 reported in another recent study (Gasperin, et al. 2014). The interrelationship between FSH
 430 and BMP regulation has been previously reported (Miyoshi, et al. 2006, Shi, et al. 2009, Shi,
 431 et al. 2010), and the decline in pituitary FSH secretion initiating the dominant follicle
 432 selection process would, therefore, appear to be temporally related to the decline in
 433 BMPR1B expression on the granulosa cell surface. The low levels of receptor expression in
 434 the small antral follicles of older patients with reduced ovarian reserve suggest a possible
 435 cause of poor quality follicles and oocytes typical of older patients. Oocytes surrounded by
 436 cumulus cells with greater levels of *BMP15* mRNA were shown to have an increased

437 pregnancy rate after IVF (Li, et al. 2014), and reduced apoptosis (Hussein, et al. 2005).
 438 Moreover, an association between high levels of BMP15 in the follicular fluid and oocyte
 439 quality has been reported (Li, et al. 2014, Wu, et al. 2007).

440 *4.2. BMPR1B down-regulation and the maturation of pre-ovulatory follicles*

441 The degenerative ageing of granulosa BMPR1B density is highlighted by the observation
 442 that 40+ y patients (groups B & C) with a favourable ovarian reserve for age exhibit a
 443 pattern of declining receptor density with follicle size, whereas the pattern in the 40+ y
 444 patients (groups D & E) with reduced ovarian reserve is reversed (Fig. 7). Similar ovarian
 445 ageing was found in the 35-39 y B & C group with the same steady increasing density in the
 446 reduced ovarian reserve D & E group (Fig. 6). In addition, evidence of ovarian ageing was
 447 seen in the youngest patients with a severely reduced for age ovarian reserve (AFC group C),
 448 where the receptor density was increased substantially (Fig. 4). The over-expression of
 449 BMPR1B was also present in the oldest patients with severe ovarian depletion (AFC E; Fig
 450 7A). In the 31-34 y patient group with an A ovarian reserve, the receptor density increased in
 451 the largest follicles followed by a general decrease in the poorer ovarian reserve patients for
 452 that age group (Fig. 5A). As age increases, and the ovarian reserve declines, fewer follicles
 453 are stimulated; hence, the extra-large follicles are rare. In the younger patients of 31-34 y, it
 454 would be expected that a 24+ mm follicle would be common; however, none were analysed.
 455 The reversal of receptor density provides evidence of a fundamental shift in granulosa
 456 BMPR1B receptor density with ovarian ageing. High levels of BMPR1B in pre-ovulatory
 457 follicles would promote oestrogen synthesis and inhibit progesterone synthesis, which could
 458 potentially suppress maturation of the follicle (Otsuka 2010, Shimasaki, et al. 1999).

459 *4.3. Could the apparent effect of ovarian ageing on BMPR1B receptor density be due* 460 *to different degrees of rFSH stimulation in the treatment cycle?*

461 Patients with declining ovarian reserves are prescribed greater doses of rFSH and this could
 462 potentially confound the interpretation of the present observation that ovarian ageing affects
 463 granulosa BMPR1B receptor density. However, when 'like-with-like' comparisons were

made, with only those patients prescribed similar doses of rFSH included in the analyses, the effect of ovarian ageing on receptor expression persisted. The changes observed in BMPR1B density are therefore, unlikely to be attributable to the degree of rFSH stimulation that the patient received during a treatment cycle, at least within patients of a similar chronological age. In support of this, unpublished findings from one of our laboratories (PGK) have indicated that treatment of cultured bovine granulosa cells with FSH promotes a marked increase in CYP19A1 mRNA expression and E2 secretion but has no effect on BMPR1A, BMPR1B or BMPR2 mRNA expression (C Glistner and PG Knight, unpublished observations).

4.4. Ovarian gonadotrophin surge attenuating factor (GnSAF) and BMPR1B receptor density during folliculogenesis: is there a link?

The BMPs have been described as inhibitors of the LH surge and luteinisation (Otsuka 2010, Shimasaki, et al. 1999). GnSAF is an uncharacterised follicular-derived factor purported to reduce GnRH-induced pituitary LH secretion (Dimitraki, et al. 2014). An inverse relationship between GnSAF activity and follicle size has been reported (Fowler, et al. 2001). Furthermore, the age-related decline in ovarian reserve was associated with reduced GnSAF activity during follicle development (Martinez, et al. 2002). Could this be linked to the somewhat similar effect of age on granulosa BMPR1B receptor density shown in the present study? In the current study the observed changes in granulosa BMPR1B density in the young and older patients mimic the changes in GnSAF activity described by Martinez et al. (2002). Martinez et al. (2002) concluded that the GnSAF bioactivity prevented the premature onset of the LH surge, which bears comparison with the proposed role of BMPs as an inhibitor of luteinisation as evidenced by others (Otsuka 2010, Shimasaki, et al. 1999). In this context, it should be mentioned that both BMP ligands and receptors are expressed at the anterior pituitary gland level and BMP signalling has been implicated in the regulation of gonadotrophin production (Nicol et al 2008). Thus, it is tempting to speculate that ovarian GnSAF bioactivity, as yet uncharacterised, might actually

491 be attributed to BMPs synthesized and secreted by ovarian follicles. Clearly, further detailed
492 experiments would be required to evaluate the tenability of this suggestion.

493 *4.5. Conclusion*

494 Taken together, the results demonstrate the disrupting effect that ageing-induced depletion of
495 the ovarian reserve has on granulosa BMPR1B receptor density in antral follicles. Age-
496 induced depletion is associated with a loss of the biphasic down-regulation of granulosa
497 BMPR1B density during follicle development. The findings extend previous research by the
498 comprehensive nature of the range of follicle sizes and age groups studied, together with
499 measurement of the translated mature, BMPR1B protein as opposed to measurement of
500 receptor expression at the mRNA level (Ascoli, et al. 2002). Further work is needed to
501 confirm the identity of the locally-produced TGF β family ligand(s) (BMP2, BMP4, BMP6,
502 BMP 7, and BMP15) whose signalling may either promote, or be impacted by this change in
503 BMPR1B receptor density on the granulosa cell surface, and also to explore the
504 consequences of altered signalling.

505

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509 **Authors' roles**

510 S.L.P.R. performed the experiments, analysed and interpreted the data, and wrote the
511 manuscript. All authors contributed to the study design, manuscript revision, and final
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517 **Conflict of interest**

518 The authors declare that there is no conflict of interest that could be perceived as prejudicing
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520

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